

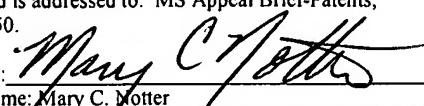


IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Allen, Keith D. Examiner: Qian, Celine X  
Serial No.: 10/005,467 Group Art Unit: 1636  
Filed: December 4, 2001 Docket: R758/75658.295  
Confirmation No.: 7217  
Title: Transgenic Mice Containing PTP36 Tyrosine Phosphatase Gene Disruptions

Express Mail Number: EV780369175US  
Date of Deposit: March 30, 2006

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By:   
Name: Mary C. Motter

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PATENT TRADEMARK OFFICE

Sir:

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- Transmittal Sheet
- Reply Brief
- Applicant claims small entity status. See 37 CFR 1.27
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Alexandria, VA 22313-1450

**REPLY BRIEF**

**Response to Examiner's Answer**

Sir:

In regard to the above-referenced application, Appellant submits this Response to the Examiner's Answer, mailed February 13, 2006.

**I. REAL PARTY IN INTEREST**

A statement appears in the Appeal Brief filed November 11, 2005.

**II. RELATED APPEALS AND INTERFERENCES**

A statement appears in the Appeal Brief filed November 11, 2005.

**III. STATUS OF CLAIMS**

A statement appears in the Appeal Brief filed November 11, 2005.

**IV. STATUS OF AMENDMENT**

A statement appears in the Appeal Brief filed November 11, 2005.

**V. SUMMARY OF CLAIMED SUBJECT MATTER**

A statement appears in the Appeal Brief filed November 11, 2005.

**VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

A statement appears in the Appeal Brief filed November 11, 2005, as modified by the Examiner's Answer.

**VII. ARGUMENT**

**A. Response to Examiner's Answer**

The majority of the Examiner's arguments have been previously made and do not warrant further response from Applicant. However, a few issues will be addressed herein.

With regard to the asserted utility of identifying agents capable of affecting a phenotype of the claimed mouse, the Examiner argues "[i]n other words, why would a skilled artisan wish to identify such agent?" *See* Examiner's Answer at page 4.

The claimed mice manifest androgenization, also known as hyperandrogenism, a condition that may ultimately lead to life-threatening cardiovascular problems and metabolic disorders. *See* Appeal Brief, pages 23-24. Thus, one skilled in the art would be interested in developing methods of treating androgenization by identifying agents capable of interacting with and modifying the activity of the PTP36 gene and its expression product.

With regard to the asserted utility of identifying agents as useful therapeutic agents for treating conditions associated with a disruption of the PTP36 gene, the Examiner argues that “the specification does not disclose what kind of conditions is associated with a disruption or other mutations of the PTP36 gene.” *See Examiner’s Answer at page 5.*

According to the specification, the claimed mice demonstrated androgenization:

The homozygous female mice did not have demonstrable mammary gland tissue or had only a few mammary ducts on deeper levels. The homozygous female mice also contained the presence of keratin in the uterine horns; more specifically, keratinous debris was detected, but without concomitant squamous metaplasia, suggestive of passage of keratin from the vagina through a dilated cervix, indicating cervical relaxation. Increased anogenital distance in homozygous mutant females was also detected. The combination of lack of mammary growth and cervical relaxation are suggestive of a hormonal imbalance. Hormones having an effect on reproductive and/ or mammary tissues during development include estrogens, progesterones, growth hormone, thyroxine and insulin. **The phenotypes observed are consistent with androgenization of the mutant female mice.**

(page 49, lines 14-23) (emphasis added) *See also, Appeal Brief at page 16.* Thus, the specification discloses conditions associated with disruption of the PTP36 gene.

With regard to the asserted utility of identifying agents having an effect on PTP36 expression or function, the Examiner argues that the specification does not disclose 1) how to use a mouse or cell that does not express PTP36; 2) how to use a heterozygous PTP36 knockout mouse; and 3) how to use such identified agent.

According to the specification (page 3, lines 3 through page 4, line 19):

The present invention further provides non-human transgenic animals and methods of producing such non-human transgenic animals comprising a disruption in a PTP36 gene. *The transgenic animals of the present invention include transgenic animals that are heterozygous and homozygous for a mutation in the PTP36 gene.* In one aspect, the transgenic animals of the present invention are defective in the function of the PTP36 gene. In another aspect, the transgenic animals of the present invention comprise a phenotype associated with having a mutation in a PTP36 gene.

*The present invention also provides methods of identifying agents capable of affecting a phenotype of a transgenic animal. For example, a putative agent is administered to the transgenic animal and a response of the transgenic animal to the putative agent is measured and compared to the response of a "normal" or wild-type mouse or, alternatively, compared to a transgenic animal control (without agent administration).* The invention further provides agents identified according to such methods. The present invention also provides methods of identifying agents useful as therapeutic agents for treating conditions associated with a disruption of the PTP36 gene.

*The present invention further provides a method of identifying agents having an effect on PTP36 expression or function.* The method includes administering an effective amount of the agent to a transgenic animal, preferably a mouse. *The method includes measuring a response of the transgenic animal, for example, to the agent and comparing the response of the transgenic animal to a control animal, which may be, for example, a wild-type animal or, alternatively, a transgenic animal control. Compounds that may have an effect on PTP36 expression or function may also be screened against cells in cell-based assays, for example, to identify such compounds.*

The invention also provides cell lines comprising nucleic acid sequences of a PTP36 gene. Such cell lines may be capable of expressing such sequences by virtue of operable linkage to a promoter functional in the cell line. Preferably, expression of the PTP36 gene sequence is under the control of an inducible promoter. Also provided are methods of identifying agents that interact with the PTP36 gene, comprising the steps of contacting the PTP36 gene with an agent and detecting an agent/PTP36 gene complex. Such complexes can be detected by, for example, measuring expression of an operably linked detectable marker.

The invention further provides methods of treating diseases or conditions associated with a disruption in a PTP36 gene and, more particularly, to a disruption in the expression or function of the PTP36 gene. *In a preferred embodiment, methods of the present invention involve treating diseases or conditions associated with a disruption in the PTP36 gene's expression or function, including administering to a subject in need, a therapeutic agent that effects PTP36 expression or function.* In accordance with this embodiment, *the method comprises administration of a therapeutically effective amount of a natural, synthetic, semi-synthetic, or recombinant PTP36 gene, PTP36 gene products or fragments thereof as well as natural, synthetic, semi-synthetic or recombinant analogs.*

*The present invention also provides compositions comprising or derived from ligands or other molecules or compounds that bind to or interact with PTP36, including agonists or antagonists of PTP36. Such agonists or antagonists of PTP36 include antibodies and antibody mimetics, as well as other molecules that can readily be identified by routine assays and experiments well known in the art.*

*The present invention further provides methods of treating diseases or conditions associated with disrupted targeted gene expression or function, wherein the methods comprise detecting and replacing through gene therapy mutated PTP36 genes.*

The specification further provides (page 17, lines 29 through page 18, line 12):

More particularly, using the animal models of the invention, specifically, transgenic mice, methods of identifying agents, including compounds are provided, preferably, on the basis of the ability to affect at least one phenotype associated with a disruption in a PTP36 gene. *In one embodiment, the present invention provides a method of identifying agents having an effect on PTP36 expression or function. The method includes measuring a physiological response of the animal, for example, to the agent, and comparing the physiological response of such animal to a control animal, wherein the physiological response of the animal comprising a disruption in a PTP36 as compared to the control animal indicates the specificity of the agent. A "physiological response" is any biological or physical parameter of an animal that can be measured.* Molecular assays (e.g., gene transcription, protein production and degradation rates), physical parameters (e.g., exercise physiology tests, measurement of various parameters of respiration, measurement of heart rate or blood pressure, measurement of bleeding time), and cellular assays (e.g., immunohistochemical assays of cell surface markers, or the ability of cells to aggregate or proliferate) can be used to assess a physiological response.

The specification additionally provides (page 50, lines 10-13):

*LacZ Reporter Gene Expression.* In general, *tissues from 7-12 week old heterozygous mutant mice were analyzed for lacZ expression.* Organs from heterozygous mutant mice were frozen, sectioned (10 µm), stained and analyzed for lacZ expression using X-Gal as a substrate for beta-galactosidase, followed by a Nuclear Fast Red counterstaining.

Thus, the specification discloses 1) how to use a mouse or cell that does not express PTP36 to identify agents; 2) how to use a heterozygous PTP36 knockout mouse to identify agents and for PTP36 gene expression analyses; and

3) how to use identified agents to treat diseases and conditions associated with PTP36 gene expression or disruption.

The Examiner argues that “[a]t the time of filing, the phenotype of the transgenic mouse is considered unpredictable.” *See Examiner’s Answer at page 9.*

Although the issue raised by the Examiner appears to have been previously raised and waived (see Office Action of June 3, 2003; Office Action of December 31, 2003), Applicant will address the Examiner’s points.

Among the additional arguments made, the Examiner argues that phenotype is influenced by genetic background, citing Sigmund. *See Examiner’s Answer at page 9.*

Sigmund discusses guidelines with regard to minimizing “potential” background effect. As pointed out by Sigmund, ideally the knockout mice are compared with mice of the same background; and preferably, the mice are backcrossed to reduce background effect (page 1426-7). Sigmund also suggests using littermates as controls (page 1428). Applicant follows these guidelines. According to the specification (Example 1):

ES cells derived from the 129/OlaHsd mouse substrain were used to generate chimeric mice. F1 mice were generated by breeding with C57BL/6 females. ***F2 mutant mice were produced by intercrossing F1 heterozygous males and females.***

***Wild-type control mice, as well as heterozygous and homozygous mutant mice, were evaluated by the following examinations or tests:***

- Physical examinations.
- Necropsy, including body length, body weight, and organ weight measurements.
- Histological examination of tissues and organs.
- Bone marrow section evaluations.

- Complete blood counts and differentials.
- Clinical chemistry panels.
- Behavioral tests.

In summary, the transgenic mice exhibited one or more of the following phenotypes:

- Histopathology : Uterine dilation; keratin in the uterine lumen; absence of or reduction in mammary gland tissue in homozygous mutant females
- Physical Examination: Increased anogenital distance in homozygous mutant females

The homozygous female mice did not have demonstrable mammary gland tissue or had only a few mammary ducts on deeper levels. The homozygous female mice also contained the presence of keratin in the uterine horns; more specifically, keratinous debris was detected, but without concomitant squamous metaplasia, suggestive of passage of keratin from the vagina through a dilated cervix, indicating cervical relaxation. Increased anogenital distance in homozygous mutant females was also detected. The combination of lack of mammary growth and cervical relaxation are suggestive of a hormonal imbalance. Hormones having an effect on reproductive and/ or mammary tissues during development include estrogens, progesterones, growth hormone, thyroxine and insulin. The phenotypes observed are consistent with androgenization of the mutant female mice.

*When compared to age- and gender-matched controls, all three homozygous mutant females also had marginally increased body weights, and liver, spleen and thymus gland weights. The organ to body weight ratios were also slightly increased for the liver in all three homozygous mutant females and for the spleen in one of three homozygous mutant females.*

Thus, as is practiced in the art, the claimed mice were compared with control mice - i.e., mice having the same background.

With regard to the so-called hitchhiker effect discussed by Sigmund, the Examiner cannot presume a general proposition based on a rare phenomenon. According to the MPEP:

Office personnel must establish *that it is more likely than not that one of ordinary skill in the art would doubt* (i.e., "question") the truth of the statement of utility. . . . To do this, *Office personnel must provide evidence sufficient to show that the statement of asserted utility would be considered "false"* by a person of ordinary skill in the art.

(MPEP 2107.02, III(A)(emphasis added). This burden has clearly not been satisfied.

According to Wolfer *et al.*,: "the possibility exists that an apparent effect of a null mutation could be due to a flanking 129 gene. Generally, the problem is disregarded because it imposes control strategies deemed costly, and because the statistically expected number of confounding flanking genes is relatively low" (emphasis added) (2002, *TRENDS in Neuroscience*, 25:336-340; page 336)(Exhibit W). Thus, hitchhiker alleles are a rare phenomenon.

The Examiner argues that the transgene expression and physiological consequences of transgene products are not always accurately predicted in transgenic mouse studies, citing Wall. See Examiner's Answer at page 9.

The Examiner cites Wall out of context. Wall discusses issues associated with expression of transgenes in livestock and that experimentation should take place in the species of interest. Wall states "[t]hat is because transgene expression and the physiological consequence of transgene products in livestock are not always accurately predicted in transgenic mouse studies." Wall at page 61-62.

First, Applicant is claiming a mouse, not livestock. Second, Applicant is claiming a knockout mouse, *i.e.*, a mouse with a null allele, not a transgenic mouse expressing a transgene. Wall is therefore irrelevant to the claimed invention.

The Examiner cites Jacks for demonstration of an unexpected phenotype in Rb knockout mice. Tumors were found to arise not in the retinas, as in humans, but rather in the pituitary.

It is clear that the Rb gene is associated with tumor growth, both in mice and in humans. Thus, the phenotype of the mouse correlates with the function of the gene in humans. The difference is one of degree (location of tumors) not one of kind. This does not support the Examiner's position that knockout mice phenotypes are unpredictable.

The Applicant has created a knockout mouse wherein the PTP36 gene is disrupted. This mouse was compared with control mice of the same background. Since homozygous, heterozygous and control mice are littermates, the only difference between the knockout mice and the control mice is the absence of a functional PTP36 gene. It is clear that the androgenization phenotype, present in the knockout mice but not in the wild-type controls, is a result of the PTP36 gene disruption. Thus, one skilled in the art would accept that the PTP36 gene is involved in sexual development and fertility.

As observed by Doetschman (Exhibit H), one clearly skilled in the art, the phenotypes observed in mice do correlate to gene function:

The conclusions will be that the knockout phenotypes do, in fact, provide accurate information concerning gene function, that we should let the unexpected phenotypes lead us to the specific cell, tissue, organ culture, and whole animal experiments that are relevant to the function of

the genes in question, and that the absence of phenotype indicates that we have not discovered where or how to look for a phenotype.

*See also, Appeal Brief at pages 13-14.*

With regard to the correlation between murine and human gene function, Sands (Exhibit P) states: “[a]fter a decade of using mouse knockouts, the data on their predictive power in drug discovery is irrefutable.” *See also, Appeal Brief at page 22.*

Applicant further directs attention to a recent NIH press release, wherein the NIH announced it was accessing Deltagen’s data derived from its analysis of the mice:

BETHESDA, Md., Wed., Oct. 5, 2005 - *The National Institutes of Health (NIH) today announced contracts that will give researchers unprecedented access to two private collections of knockout mice, providing valuable models for the study of human disease and laying the groundwork for a public, genome-wide library of knockout mice.*

Under terms of three-year contracts jointly funded by 19 NIH institutes, centers and offices, *Deltagen Inc.* of San Carlos, Calif., and Lexicon Genetics Incorporated of The Woodlands, Texas will provide NIH and its scientific partners *with access to extensively characterized lines of mice in which a specific gene has been disrupted, or "knocked out."* In the first year of the contract, NIH will expend about \$10 million to acquire about 250 lines of knockout mice.

For each mouse line, the contractors will provide not only the mouse line itself, but also detailed, objective data on the impact of the specific gene deletion on the mouse’s phenotype, which includes appearance, health, fitness, behavior, ability to reproduce, and radiological and microscopic data. *Such comprehensive information on such a large group of mice has never been available to public sector researchers, and is expected to greatly accelerate efforts to explore gene functions in health and disease.*

*“Our decision to procure these knockout mouse lines and data and make them available to the research community will yield tremendous*

*benefits, both in the short and long terms," said NIH Director Elias A. Zerhouni, M.D. "This trans-NIH initiative will place important mouse models into the hands of researchers, speeding advances in the understanding of human disease and the development of new therapies. It also represents a significant step in the direction of launching an international project to systematically knock out all genes in the mouse."*

Since the early 1980s, when recombinant DNA technology was used to create the first such animals, *knockout mice have proven to be one of the most powerful tools available to study the function of genes and to create mouse models of human disease.* Researchers have produced knockout mice with characteristics similar to humans suffering from a wide range of disorders, including cancer, heart disease, neurological disorders and even obesity.

(Exhibit I). The claimed invention is one of 750 lines of mice contained within Deltagen's DeltaBase collection. Under the terms of its contract, the NIH has a three-year option to obtain access to all of these lines of mice. Under its initial budget, the NIH has elected to access approximately 125 lines of these mice (which does not yet include the claimed mouse), and has approximately 30 months to select additional lines of mice. Thus, the NIH has initially spent approximately five million dollars (\$5,000,000.00) to access a subset of Deltagen's DeltaBase collection. Applicant submits that the NIH would not have expended public funds if they did not believe that the phenotypes were a result of the disruption.

In fact, with regard to Applicant's methods and knockout mice, the NIH has publicly stated (Exhibit I):

*The process used by NIH to select the mouse lines involved a rigorous scientific review process that evaluated information on the knocked out gene, the reliability of the method used to produce the knockout, and whether the mouse line possesses a "reporter" gene, which enables researchers to analyze the pattern of the knockout gene's expression in various mouse tissues.*

Finally, Applicant notes that collectively, Merck & Co., Pfizer Inc. and GlaxoSmithKline have spent more than sixty million dollars (\$60,000,000.00) to access Deltagen's DeltaBase. *See Appeal Brief at page 19.* All of these companies performed extensive due diligence on Deltagen's technology prior to entering into subscription contracts with Deltagen. Additionally, every one of the mice contained within DeltaBase, including the claimed invention, has been ordered by at least one large pharmaceutical company. These companies would not have spent their research dollars on these mice and data if they were not satisfied that the phenotypes were a result of the disruption.

Thus, large pharmaceutical companies, including Merck, Glaxo, and Pfizer, among many others, and the NIH have accepted the methods used by Deltagen as reliable in creating their knockout mice. Applicant submits that the methods and models developed by Applicant have been clearly validated by those skilled in the art. The Patent Office should not require a standard higher than those skilled in the art.

With regard to citation of an NIH report (Exhibit A) and Austin (Exhibit B), the Examiner argues that the Exhibits were not available until 2004 and cannot be used to establish what was "well-established" at the time of filing. *See Examiner's Answer at pages 12-13.*

The references are not being cited to support a post-filing assertion of utility. The goal of determining gene function is clearly set forth in the specification. *See, for example,* specification, page 2, lines 8-11. Austin supports this statement of utility. Moreover, courts have accepted post-filing activities to support an asserted utility. For example, in *In re Brana*, the application's filing date was June 30, 1988. The applicants relied on an affidavit submitted June 19,

1991 to provide evidence of the compounds activity, a date well after the filing date. The Federal Circuit noted:

Enablement, or utility, is determined as of the application filing date [citations omitted]. *The Kluge declaration, though dated after applicants filing date, can be used to substantiate any doubts as the asserted utility since this pertains to the accuracy of a statement already in the specification.* [citations omitted] It does not render an insufficient disclosure enabling, but instead goes to prove that the disclosure was in fact enabling when filed (i.e., demonstrated utility).

(*In re Brana*, 51 F.3d 1560, 1567, n.19). As the asserted utility of determining gene function was already in the specification, Austin and the NIH citation should be considered by the Examiner as evidence supporting the utility of the claimed invention.

In summary, the asserted utilities of using the PTP36 knockout mouse to (i) study the function of the PTP36 gene; (ii) to study the role of PTP36 in androgenization and identify therapeutic treatments for androgenization; (iii) study the role of PTP36 in cancer and identify therapeutic treatments for cancer; (iv) study specificity and side-effects of therapeutics targeting the PTP36 gene; and (iv) study expression of the PTP36 gene using lacZ expression analysis, are well established, credible, substantial and specific. This conclusion is supported by (i) the well recognized use of knockout mice for gene function study and therapeutic drug development; (ii) the delivery of the claimed mouse to at least one large pharmaceutical company; (iii) the incorporation of the data derived from analyses of the claimed mouse into a commercially licensed database; and (iv) the validation of Deltagen's methods and models as exemplified by the NIH's decision to contract for an option to obtain access to the mice contained within that database, including the claimed mouse.

**VIII. CLAIMS APPENDIX.**

A copy of the claims appears in the Appeal Brief filed November 11, 2005.

## IX. EVIDENCE APPENDIX

Enclosed, please find copies of the following additional references:

### Exhibit W

Wolfer et al., 2002, *TRENDS in Neuroscience*, 25:336-340

**X. CLOSING REMARKS**

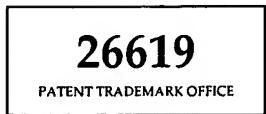
For the foregoing reasons, Appellant submits that the rejection of claims 28-32, 37, 47 and 53-57 under 35 U.S.C. § 101 for lack of utility is improper; and that the rejection of claims 28-32, 37, 47 and 53-57 under 35 U.S.C. § 112, first paragraph, for lack of enablement is improper.

It is believed that no other fees are due with this Appeal Brief, Response to Examiner's Answer. If this is in error, please charge any additional fees to Deposit Account No. 502775.

This constitutes a request for any needed extension of time under 37 C.F.R. § 1.136(a) and an authorization to charge all fees therefore to deposit account No. 502775 if not otherwise specifically requested.

Respectfully submitted,

3-30-06  
Date



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## Techniques &amp; Applications

# Knockout mice: simple solutions to the problems of genetic background and flanking genes

David P. Wolfer, Wim E. Crusio and Hans-Peter Lipp

**Inducing null mutations by means of homologous recombination provides a powerful technique to investigate gene function and has found wide application in many different fields. However, it was realized some time ago that the specific way in which such knockout mutants are generated can be confounding, making it impossible to separate the effects of the induced null mutation from those of alleles originating from the embryonic stem cell donor. In addition, effects from null mutations can be altered on different genetic backgrounds. Here we present some simple breeding strategies to test for flanking gene effects that are compatible with the recommendations of the Banbury Conference on Genetic Background in Mice and with common practices of creating and maintaining mouse knockout lines.**

Recently, it has been realized that the experimental design of many studies in which genes were 'knocked-out' by means of homologous recombination are affected by a confounding factor: the flanking-gene problem [1,2]. Briefly, the problem is a consequence of the fact that the embryonic stem (ES) cells used in many such experiments are derived from substrains of 129 INBRED (see Glossary) mice [3]. A CHIMERA in which such ES cells populate the germ line will transmit not only the induced null mutation, but also the 129 genetic background, in their germ lines. Such males are usually mated to females from another inbred strain (generally C57BL/6), and the F1 hybrid offspring are mated with their siblings to produce the F2 generation. As a result, animals from this F2 generation will segregate not only for the induced null mutation and its wild-type ALLELE, but also for any other alleles at loci where the parental strains differ. When comparing null mutants with wild-type animals, most of these segregating genes will not pose any serious problems, as their distributions will be independent from the null mutation. This is different, however, for any genes linked to the targeted gene. Here, a so-called linkage disequilibrium

## Glossary

- Allele:** one of two or more forms of a gene, reflecting intraspecies variance at a particular gene locus. An organism is homozygous for a gene if the alleles are identical, and heterozygous if they are different.
- Chimera:** when embryonic stem (ES) cells from a donor strain (e.g. 129) harboring targeted mutations are injected into blastocysts of a recipient strain (e.g. C57BL/6) and implanted into foster mothers, chimeras result. The chimeric animals that are born carry a conglomerate of cells from both strains. Some implanted ES cells differentiate into germ-line cells transmitting the genome (including mutation) of the donor strain, and chimeras can, thus, be mated to generate mutant mouse lines.
- Co-isogenic strains:** two inbred strains that carry different alleles of only one gene, and that are otherwise genetically identical [a]. Co-isogenic strains can arise through spontaneous mutation or through the artificial introduction of a mutation (e.g. a targeted null mutation or a transgene) into an inbred strain (e.g. by backcrossing chimeras to ES donor strain).
- Congenic strains:** strains obtained by repeatedly backcrossing mice heterozygous for a recognizable mutation (as evidenced by PCR or particular phenotypes) to an inbred strain. After ten generations, the contribution of genes from the donor strain that are not linked to the selected locus will be, on average, <0.1% [a].
- Dominant; recessive:** these terms indicate how phenotypes are inherited. A recessive phenotype is one that occurs only when an animal is homozygous for a particular allele. A phenotype is dominant if it is detectable when an animal is heterozygous for the allele. Single genes can be dominant for some phenotypes but recessive for others.
- Genetic drift:** in smaller populations, the proportion of animals heterozygous for a particular gene is reduced progressively even under conditions of chance mating, because germ cells transmit only one allele and the other allele is lost. In a population, the loss of alleles is stochastic and increases steadily. Thus, separate lines of mice bred from a common F2 cross between two inbred strains will drift apart genetically with increasing numbers of generations and will show systematic phenotypic differences unrelated to any targeted mutation.
- Haplosufficient:** the amount of gene-product generated by one functional allele is sufficient for normal gene function. On the phenotypic level this results in the mutation being recessive. The opposite situation is haploinsufficiency, where the amount of gene-product generated by one functional allele is insufficient for normal function and the phenotype related to the mutation is dominant [b].
- Hybrid vigor:** crossing two inbred mouse strains results in an F1 progeny that is isogenic, but heterozygous, for all alleles creating strain-specific deficiencies. Typically, such F1 mice are active, viable, able to out-perform both parental strains in many physiological and behavioral parameters and show minimal inter-individual differences.
- Inbred:** a strain of mice is considered 'inbred' if the mice have been brother-sister mated (sib-mated) for at least 20 generations [c]. These mice are genetically identical (isogenic) – that is, their genes are homozygous at all loci (with the exception that males and females differ for any genes on the Y chromosome).
- Phenotype:** any measurable characteristic of an organism. A phenotype is the result of interactions between genes and the environment.

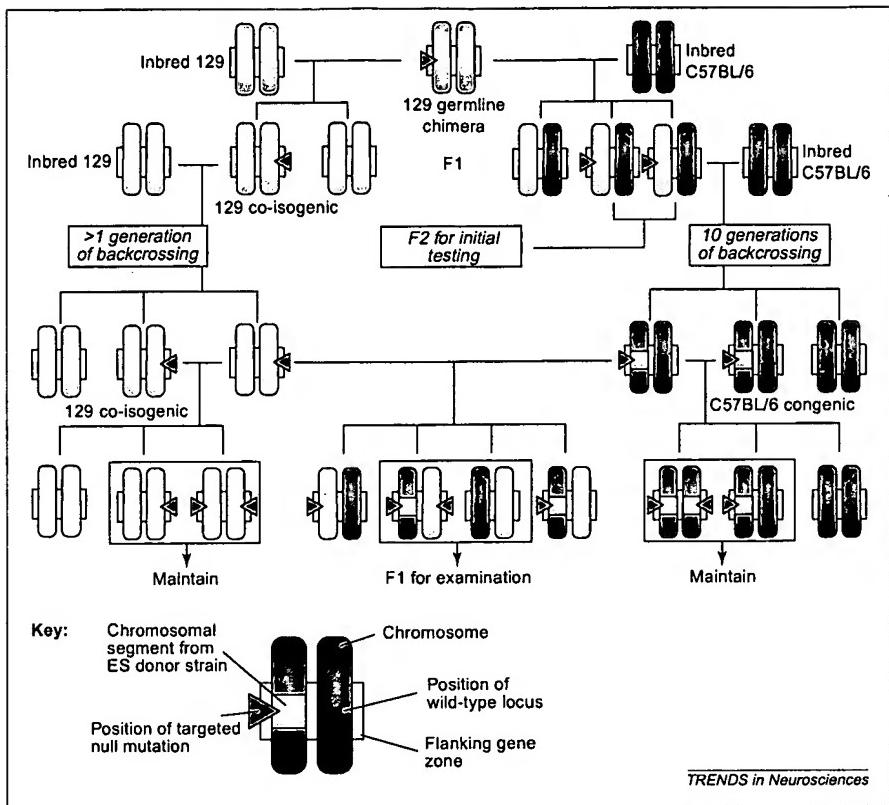
## References

- International Committee on Standardized Genetic Nomenclature for Mice (1994) Rules and guidelines for genetic nomenclature in mice. *Mouse Genome* 92, 7–32
- Crusio, W.E. (2002) 'My mouse has no phenotype'. *Genes Brain Behav.* 1, 71
- Green, E.L. (1966) *Biology of the Laboratory Mouse*, Dover Publications

will exist, because the null mutation will be flanked by two 129-derived alleles, and the wild-type locus flanked by two C57BL/6-derived alleles (Fig. 1). Therefore, the possibility exists that an apparent effect of a null mutation could be due to a flanking 129 gene. Generally, the problem is disregarded because it imposes control strategies deemed costly, and because the statistically expected number of confounding flanking genes is relatively low. However, active search for flanking-gene effects has indeed revealed candidate cases [4–6].

## Interactions with genetic background and environment

Another possible complication in gene-knockout experiments is the fact that the PHENOTYPE resulting from a null mutation can depend on the general genetic background of mouse strains used for this research. Thus, congenic strains carrying the same null mutation can sometimes show widely divergent phenotypes, depending on the genotype of the recipient strain [7,8]. Recent examples are the findings of Mineur *et al.* [9] and Ivancic and Greenough [10].



They described opposite effects of a null mutation in the *Fmr1* gene, reporting an increase in the size of the hippocampal intra- and infrapyramidal mossy fiber terminal field when the mutation was on an FVB background, but a decrease when the mutation was on a C57BL/6J background. Finally, it should

be realized that possible effects of a null mutation can also depend on the general laboratory environment [11] or on the particular behavioral testing method used [12,13]. However, we will concentrate on the genetic problems associated with targeted-gene experiments.

#### Box 1. Recommendations of the Banbury Conference on genetic background in mice [a]

##### General

- Reports of genetic experiments must include a detailed description of the genetic background.
- The genetic background should not be so complex as to preclude others from reproducing and expanding the reported experiments.
- The use of a common genetic background would facilitate the comparison of results across experiments and among laboratories.

##### Line maintenance

- Mutations should be maintained by backcrossing resulting in congenic or co-isogenic lines, preferably on both C57BL/6 and 129 backgrounds because these strains are used most widely.
- It is a faulty strategy to maintain separate mutant and control lines by continuous inbreeding of homozygous individuals starting from the original F2 generation, as this will entail genetic drift and, with it, phenotypic line differences unrelated to the targeted mutation.

##### Phenotypic characterization

- For the first characterization of a mutation, the F2 obtained by crossing the chimera to C57BL/6 and then intercrossing their heterozygous offspring offers a reasonable compromise between the demands of time and the rigorous control of genetic background.
- In the long term, it is recommended that mutants be analyzed on a defined hybrid F1 background, obtained by crossing two congenic or co-isogenic lines.

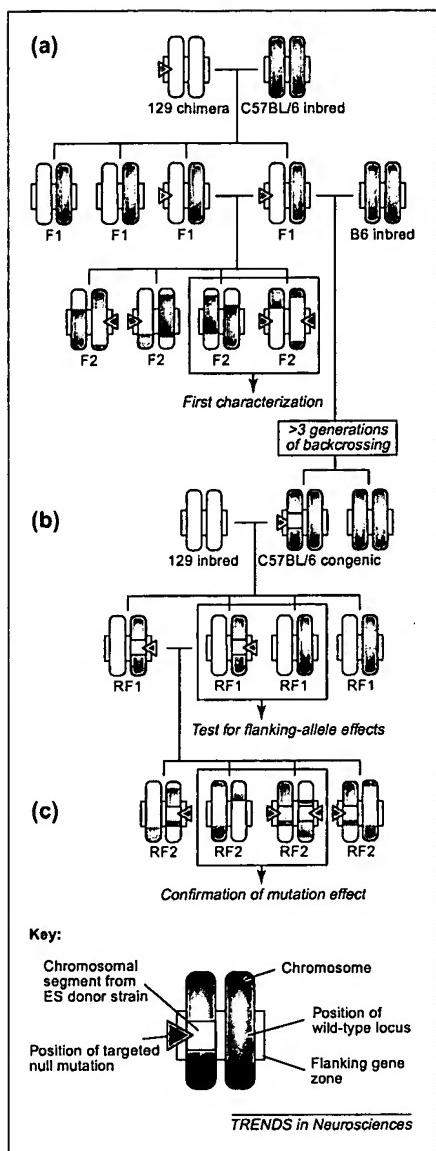
##### Reference

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**Fig. 1.** Breeding strategies recommended by the Banbury Conference for maintenance of targeted mutations in congenic and co-isogenic lines, and for later production of genetically homogeneous F1-hybrid test samples [3]. The chromosome bearing the targeted locus is shown with background alleles originating from the embryonic stem (ES) cell donor strain (in this case, 129) in white, and those from C57BL/6 in blue (the scheme can also be applied to other strain combinations). During backcrossing, heterozygous mutants are repeatedly crossed with wild-type animals from an inbred strain until the strain is said to be congenic. Backcrossing of chimeras to the ES-cell donor strain establishes a co-isogenic line (this is advisable, as ES cells can accumulate genetic damage in cell culture). Because 129 substrains often breed poorly, it can prove difficult to establish a co-isogenic line. As an alternative, a 129-congenic line can be established, starting from a population with mixed background and using predominantly inbred 129 males for backcrossing. However, this procedure is also likely to co-select some C57BL/6 genes that improve breeding success. Maintaining mutations by backcrossing to two different strains allows the repeated generation of F1-test samples by crossing the two lines. F1 hybrids are isogenic, even though they are heterozygous for loci showing allelic variation between the parental strains, which reduces their phenotypic variability remarkably. In fact, many behavioral traits show less variability in hybrid mice than in inbred strains, which is helpful in solving the standardization problem [11,12,22]. In addition, F1 (and also subsequent F2 and F3 generations obtained by intercrossing) benefit from hybrid vigor. Thus, F1 hybrids represent optimal samples for phenotypic testing [23]. By contrast, the use of test mice from a single congenic or co-isogenic line is discouraged because of potentially confounding effects of the genetic background (Fig. 2); a minimal requirement is that two congenic strains are tested. Maintaining a mutation in randomly bred animals from an initial F2 generation with segregating alleles from both strains (Fig. 2a) is a faulty strategy, particularly if wild-type and mutant animals are kept as separate lines. Owing to genetic drift, this will entail many phenotypic line differences unrelated to the targeted mutation.

##### The flanking gene problem: inadequacies of the Banbury Conference recommendations

The Banbury Conference discussed breeding strategies for maintaining targeted mutations in mouse strains, and how to deal with the genetic background problem when breeding mouse samples for testing [3]. Thus, it was recommended that targeted mutations be maintained by backcrossing the initial F1 carriers with mice of two different inbred mouse strains, eventually producing CONGENIC and/or CO-ISOCENIC lines (Fig. 1, Box 1). This provides widely comparable mice that can be used for testing and, moreover, show HYBRID VIGOR. It also means that factors confounding phenotypic analysis arising from uncontrolled breeding schemes, such as GENETIC DRIFT, can be avoided. However, this strategy cannot solve the flanking gene problem. Even after backcrossing of a mutation for more than ten generations to a maintenance strain (usually C57BL/6),



the resulting C57BL/6-congenic line will still harbor a part of the chromosome from the ES donor strain that contains a variable set of flanking genes depending on number of backcrosses. During backcrossing, there is continuous selective pressure for linked 129 genes, because only individuals heterozygous for the mutation are used to breed the next generation. Even after twelve generations, the chromosome segment containing genes from the ES-cell donor strain could contain as many as 300 genes, or 1% of the genome [2]. Likewise, mating only animals with genetic markers surrounding the targeted locus (speed congenics [14,15]) will not appreciably reduce the size of this chromosomal segment, unless the markers used are

**Fig. 2.** A backcross-outcross strategy, to dissociate the effects of a targeted recessive mutation from those of flanking genes derived from the embryonic stem (ES) cell donor strain in a constitutive knockout model. The example uses the 129 strain as ES-cell donor, and assumes that C57BL/6 mice are used to generate test animals (although the scheme can also be applied to other strain combinations). Chromosomes or chromosomal segments originating from 129 mice are shown in white, those from C57BL/6 mice in blue. (a) A recessive mutation is generated by homologous recombination in ES-cells. The transmitting 129 chimera is mated to C57BL/6-inbred mice, to generate an F1 in which heterozygous offspring are intercrossed to produce an F2 generation. Homozygous F2 individuals are compared with wild-type littermates for a first phenotypic characterization [the appropriate statistical method to analyze the results would be a two-way ANOVA (analysis of variance), with genotype and litter as the main effects]. The genetic background of the F2 is largely heterogeneous, with equal contributions from the 129 and C57BL/6 genomes – except in the flanking gene zone. In this region, background alleles come from 129 in mutant animals, but come from C57BL/6 in littermate controls. Because 129 substrains often [24] (although not always [25,26]) show neuroanatomical and behavioral peculiarities [2,8,27–32], accumulation of 129 alleles in the flanking region of mutant animals could by itself produce a phenotype that is falsely attributed to the targeted mutation. Note that the C57BL/6 strain also harbors oddities, such as hearing impairments [33], enlarged ventricles [7] and poor performance in spatial-probe-trial tests [26]. Thus, using C57BL/6 ES cells does not eliminate the flanking gene problem. (b) Following the recommendations of the Banbury Conference (Fig. 1, Box 1), the mutation is maintained by backcrossing to C57BL/6 animals. This successively reduces the number of 129 alleles in the flanking region of chromosomes carrying the mutation, but does not eliminate them. In the course of backcrossing, some heterozygous individuals are outcrossed to inbred 129 mice, to generate a 'reverse F1' (RF1) generation, which differs from a 'true' F1 only in that individuals heterozygous for the mutation are all homozygous for 129 gene loci in the flanking region. The recessive mutation is, by definition, not phenotypically visible in this RF1. Thus, phenotypic differences in the RF1 that resemble the 'mutation' phenotype in the original F2 must be attributed to the flanking region, and interpreted as evidence for a contribution of homozygous recessive 129 flanking genes to the originally observed phenotype. Note that a large number of backcrosses reduces the sensitivity of this test, because 129 alleles are increasingly eliminated from the flanking region of chromosomes carrying the mutation. If the RF1 is generated too early, 129 alleles tend to predominate outside the flanking region as well as within it, which can also reduce sensitivity. The technique for creating speed congenics [14,15] is helpful to eliminate those alleles. (c) If no evidence for flanking-allele effects are found in the RF1, heterozygous mice are intercrossed to produce a 'reverse F2' (RF2), in which the mutation effect can be confirmed and dissociated from flanking-gene effects. In the RF2, the general genetic background resembles that of the original F2. In the chromosome region flanking the target locus, by contrast, most background loci will be homozygous for 129 alleles, independently of the genotype at the target locus. A contribution of flanking 129 alleles to the phenotype can be excluded if mutants in the RF2 show the same phenotypic difference from controls as in the original F2. Note that these models apply only if there is no functional interaction between targeted and flanking loci.

very close to the targeted locus and the researcher benefits from an improbably large dose of luck (i.e. obtaining cross-overs close to the targeted locus).

#### Available strategies to solve the flanking-gene problem

The flanking-gene problem might be solved by abandoning the classic strategy used to generate targeted mutations, as there are alternative models that are unaffected. For example, in so-called 'conditional knockout' models, wild-type controls can be substituted with mutants that have the mutation in the 'off'-state [16]. Alternatively, the normal phenotype of a constitutional null mutation could be rescued by re-introduction of the wild-type allele as a transgene [17]. Finally, one might use ES cells from other strains, such as C57BL/6 [18,19], to overcome some of the problems associated with the use of 129 stem cells, and backcross the chimeras to the donor strain C57BL/6. Such ES cells are now available\*, and might permit a perfect (but costly) strategy for simultaneously producing co-isogenic lines from both 129 and

C57BL/6 ES cells and testing their F1 hybrids. However, as already discussed, backcrossing alone (to C57BL/6, or to a 129-strain deemed more suitable for phenotypic testing) cannot eliminate the genetic background problem.

#### Breeding strategies

Here we present some simple breeding strategies to deal with the flanking gene problem. The idea is to confirm the mutant phenotype by comparing littermates in which the alleles at flanking genes always come from the ES-cell donor strain, be this in wild-type or mutant animals. We present two such strategies. The first is a *post-hoc* strategy, for the majority of classic constitutional knockout mice, where a RECESSIVE (HAPLOINSUFFICIENT) or incompletely DOMINANT null mutation is being conserved by backcrossing to one strain, usually C57BL/6. The second is a strategy useful for those who have created two congenic lines following the recommendations of the Banbury Conference. Two additional breeding schemes, based on other considerations, deal with less common situations.

#### The reverse F2 strategy

Commonly, the neurobehavioral effects of a recessive or incompletely dominant

\*To obtain C57BL/6 embryonic stem cells, contact K. Bürki (kbuerki@itk.unizh.ch) at the Institute of Laboratory Animal Science at the University of Zürich.

targeted mutation are first characterized in an F2 generation (Fig. 2a), usually 12–18 months after generation of chimeras. If routine backcrossing for line maintenance to a C57BL/6 strain starts at the same time as the breeding of the first mice from chimeras, a partially congenic C57BL/6 line can be created by making between four and six backcrosses (Fig. 2a). Because this C57BL/6-congenic line becomes available towards the end of the first characterization, often when a manuscript is at the draft stage, it can be conveniently used for ruling out flanking gene effects from crucial phenotypic changes before a manuscript goes to press.

C57BL/6-congenic mice are simply crossed with the ES-cell donor strain, which makes the flanking region of the resulting mutation-carrying animals homozygous for 129-derived alleles. A first comparison of these animals with wild-type F1 animals can indicate problems, if the mice heterozygous for the mutation then show similar phenotypic changes to those observed in the initial test sample (Fig. 2b). Further crossing results in a 'reverse' F2 generation, with a genetic background statistically similar to the F2 in which the phenotype was originally described (Fig. 2b,c), but which now has all flanking regions without the linkage disequilibrium (Fig. 2).

**Testing crosses between two congenic lines**  
This strategy (Fig. 3) allows verification of a mutant phenotype previously characterized in an F1 that was produced by crossing two congenic lines. The phenotype is verified in a test population where heterozygous mutants and homozygous wild-type animals are otherwise genetically identical (i.e. they are heterozygous for genes where the 129 and C57BL/6 congenics differ and homozygous for genes where they carry the same alleles). This will be true for any chromosomal segment, including the region flanking the targeted gene (Fig. 3).

#### Complete dominance

To test for flanking genes suspected to mimic a dominant mutation effect in an initial F2 sample, simple crosses are sufficient. When the mutation had been backcrossed to the ES donor strain (e.g. 129), 129-congenics can be crossed with wild types from another strain (usually C57BL/6). This will give

littermates of which 50% will be hybrid wild types, the other half being genetically identical except for the locus with the dominant mutation (as revealed by PCR). If the two groups still differ phenotypically, then the mutation must cause the difference. If they show equal phenotypes, dominant flanking alleles must be suspected.

#### Comparing two F2 populations

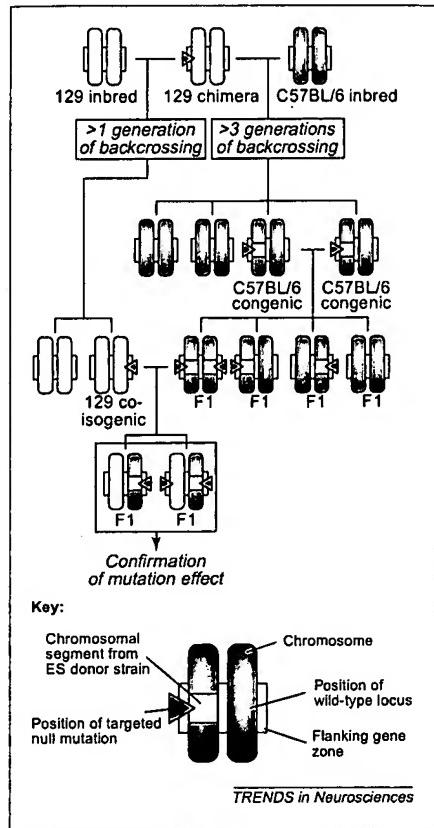
When an F2 generation is generated from a germ-line chimera and a C57BL/6 animal, one could simultaneously generate an F2 using wild-type 129 mice. Clearly, at most loci, both F2 generations will only differ because of chance variations due to segregation. The only systematic difference between the two groups will be the targeted gene, and any phenotypic difference can be ascribed to the null mutation, without confounding effects due to flanking genes [20]. However, even when large numbers of animals are used, it is evident that the statistical power of this design is not very great [21]. Further restrictions are that no littermate comparisons are possible, and that once the original chimera and any F1 animals derived from it are lost, this strategy becomes impossible.

#### Conclusions

The proposed breeding schemes require no specific technology, little resources and no more time than is already needed to comply with the recommendations of the Banbury Conference. Importantly, the 'reverse F2' strategy permits *post-hoc* testing for flanking gene effects, even when backcrossing is done only to the C57BL/6 strain (now a routine procedure in most laboratories). The only inconveniences are the replication of neurobehavioral testing some time after initial characterization, and the need to characterize phenotypically the inbred strains used to produce the knockouts. To us, this would seem good scientific practice rather than a nuisance and, compared to the risk of faulty conclusions due to confounding effects of flanking alleles, this seems a relatively small price to pay.

#### Acknowledgements

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**Fig. 3.** Breeding strategy to dissociate the effects of a targeted recessive or incomplete dominant mutation from those of flanking genes, when the recommendations of the Banbury Conference have been followed (two C57BL/6- and 129-isogenic or 129-co-isogenic lines; Fig. 1, Box 1). Heterozygous individuals of the C57BL/6-congenic line are intercrossed to produce homozygous animals that can be selected and mated with heterozygous animals of the 129-co-isogenic line. This yields a test sample in which individuals are either homozygous or heterozygous for the targeted mutation. If the mutation is recessive, comparison of homozygous and heterozygous animals should reveal different phenotypes. With respect to genetic background, these animals are 129-C57BL/6 hybrids, except in the flanking region where genes will be homozygous for 129-derived alleles because of genetic linkage to the mutation. The degree of this predominance does not depend on genotype, however, because the genotype is determined by which chromosome is inherited from the 129-co-isogenic parent. Thus, flanking genes cannot contribute to phenotypic differences between homozygous and heterozygous animals in this population. Note that minor interpretation problems will arise if a mutant phenotype that was first described in a genetically heterogeneous F2 generation fails to reproduce fully in a test population with maximized hybrid vigor (which tends to reduce slightly any mutation effect). Thus, it will remain unclear whether this failure merely reflects the difference in hybrid vigor or whether it must be interpreted as evidence for flanking gene effects.

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## Rodent models of prefrontal cortical function

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In this article, we consider whether studies in rats can provide useful information regarding the debate about the functions of the primate prefrontal cortex. At a superficial level, comparison of regional specializations within the prefrontal cortices of different species suggests functional correspondence. Unfortunately, the nature of functional specialization in primate prefrontal cortex is controversial, and data supporting the idea of homology between specific areas of rat and primate prefrontal cortex are weak. Nevertheless, we argue here that studies of the computational functions within the relatively undifferentiated prefrontal cortex of rats can shed light on processing in primate prefrontal cortex.

If any region of the cerebral cortex is unique to the evolution of primates, then the dorsolateral prefrontal cortex is likely to be it. The human dorsolateral prefrontal cortex is involved in many complex cognitive processes that have been described as 'executive' – that is, that they form a control system that coordinates cognitive sub-processes. For example, dorsolateral prefrontal cortex is thought to be involved in working memory and holding task-relevant information 'on-line' [1,2], supervisory attentional control [3], reasoning and decision-making [4] and the temporal organization of behaviour [5,6]. Despite the fact that their intellectual

functioning seems to be spared, patients with damage to frontal cortex can suffer great personal and social difficulties [7]. Compromised prefrontal function is thought to underlie the myriad of complex cognitive deficits that accompany disorders such as Alzheimer's disease [8,9], schizophrenia [10,11] and Parkinson's disease [12,13].

Preuss pointed out that there was an absence of evidence, rather than evidence of an absence, for the rat prefrontal cortex including an area homologous to primate dorsolateral prefrontal cortex [14]. Preuss also noted that it was not necessary to postulate that the rat possessed a homologous area: there are considerable